Long-Term Nonprogressive Infection with Human Immunodeficiency Virus Type 1 in a Hemophilia Cohort

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Seven long-term nonprogressors (LTNPs) have been identified in a cohort of 128 human immunodeficiency virus (HIV)-1 infected individuals with hemophilia. Studies included quantitation of virus by polymerase chain reaction, characterization of primary virus isolates in vitro, analysis of lymphocyte surface markers, and measurement of virus-specific cytotoxic T lymphocytes (CTLs). Viruses of LTNPs exhibited slow growth in vivo and in vitro. LTNPs had expansion of CD8 T cells with increased expression of HLA-DR. Intermittent HIV-1-specific CTL effector activity was detected in freshly isolated peripheral blood mononuclear cells of most LTNPs. CTL precursor frequencies were higher in LTNPs than in patients with progressive disease. Virus antigen–specific lymphoproliferation was vigorous in some LTNPs. Thus, LTNPs in this cohort have maintained remarkably low virus burdens and vigorous HIV-1–specific cell-mediated immunity over a 15-year period. The presence of expanded, activated CD8 T cells with cytotoxic effector function in the peripheral blood suggests ongoing viral replication.

More than a decade after recognizing the epidemic of human immunodeficiency virus (HIV)-1 infection in the developed world, investigators have begun to focus their attention on rare individuals who have been infected for >10 years and yet show no signs of immune suppression. It is increasingly evident that a variety of factors may influence the rate of disease progression. An understanding of the underlying reasons for long-term nonprogressive infection is likely to provide insights into the pathogenic processes involved in HIV-1 infection and control of the disease.

HIV-1 replication is poorly controlled in most infected individuals, even during the long asymptomatic period [1, 2]. Studies of the dynamics of virion production and CD4 T cell turnover have shown that immune attrition is the direct result of persistent, unrelenting viral replication [3, 4]. These insights have greatly influenced the approaches to questions of HIV pathogenesis and therapeutic management of infected people [5].

The discovery of coreceptors required for HIV-1 entry has revealed a set of host factors that may influence the rate of disease progression. Delayed disease progression is associated with sequence polymorphisms found in alleles of certain chemokine receptors and chemokines [6, 7]. The extent to which chemokine levels influence disease progression is yet to be defined [8].

Properties of the virus may also influence the rate of disease progression. Viral phenotype has been measured in a crude way with assays of syncytium induction. A syncytium-inducing phenotype has been associated with rapid loss of CD4 T cells [9]. Switches in chemokine receptor usage (R5→X4) have been seen in serial isolates from some individuals experiencing disease progression [10]. Additional phenotypes that distinguish virus isolates include the following: replication rate, infectivity, and cell-killing ability in single-cycle assays [11–14]. These could also play a significant role in influencing disease progression.

In only a minority of long-term nonprogressors (LTNPs) have viral determinants of nonprogression been identified. Uniformly defective NEF genes over the entire course of infection have been found in 1 individual (LTNP 1) from the
cohort described in this report (patient 1 in [15]). Viruses from the LTNNPs of the Sydney Blood Bank Cohort (SBBC) share deletions in the NEF gene [16], thus defining a group of unrelated individuals infected with an attenuated strain of HIV-1. An additional LTNNP was recently described to have uniformly deleted NEF alleles from 3 time points >10 years after infection [17].

Other detailed analyses have failed to show a clearly attenuating, uniform, and nonrevertible genetic defect in the viruses of LTNNPs [18, 19]. There are, however, virus isolates from LTNNPs that show unusual variability in their accessory genes. In one long-term survivor, the majority of accessory and regulatory genes were grossly defective by sequence analysis [20]. A mother and her child notable for their lack of disease progression were found to harbor viruses with an unusual level of defects and polymorphism at the C-terminal region of the VPR gene [21]. In another LTNNP in the cohort described here (LTNP 4), most viral sequences obtained over the course of infection were found to have defects in NEF by sequence analysis and/or functional studies [22]. Because viruses with intact accessory genes appear to be present yet do not become predominant in these individuals, potent negative selective pressures are likely to be operating on the viruses with fully functional alleles.

Not surprisingly, evidence suggests that immune responses may also play a major role in defining disease progression. Levels of virus-specific cytotoxic T lymphocyte (CTL) activity and CD4 lymphoproliferation have been inversely associated with viral replication and disease progression by several groups [23–26]. Demonstrations of virus escape from neutralizing antibodies and CTLs suggest that host immune responses can limit viral growth [27–29]. CTLs directed against highly conserved epitopes have been defined in LTNNPs [30, 31] and it seems probable that vigorous virus-specific immune responses contribute significantly to the development of nonprogressive HIV-1 infection.

Reports to date have presented a general picture of LTNNPs as a heterogeneous group in terms of virological and immunological characteristics [15, 18, 22, 32–37]. Detailed, comprehensive studies of LTNNPs are necessary to fully define factors that contribute to the development of disease. We provide here a summary of virological and immunological characteristics of LTNNPs in a hemophilia cohort.

Methods

Subjects. Members of this cohort study are individuals with hemophilia who have been cared for at the New England Area Hemophilia Center at UMass Memorial Health Care in Worcester, MA. Our study included 128 participants who were infected with HIV-1 before 1986 (the vast majority before 1983) through infusion of contaminated blood products and 37 participants born before 1983 who remained seronegative. The youngest infected member of the cohort was born in 1982. Infected individuals who could not be categorized into a subgroup defined by rate of disease progression because of lack of sufficient surface marker data or loss to follow-up were excluded from these analyses. All participants have given informed consent for these studies.

LTNP definition. “LTNNPs” were defined as individuals who were infected for >10 years with no signs or symptoms of HIV-1 disease, normal absolute CD4 T cell counts, and no antiretroviral therapy. Specifically required were absolute CD4 T cell counts maintained at >400/μL with a CD4 percentage >30%, or absolute CD4 T cell counts >600/μL regardless of CD4 percentage. Seven individuals met this definition when it was formulated in 1992. The course of infection for these LTNNPs through 1997 is described in the results.

We have further divided the infected members of the cohort into subgroups by the rapidity of loss of CD4 T cells. Progressors (Ps; n = 69) are individuals who by 1992 had progressed to death because of HIV-1, to an absolute CD4 T cell count <200/μL with a CD4 percentage <10%, or to an absolute CD4 T cell count <100/μL regardless of CD4 percentage. Slow progressors (SPs; n = 52) are the individuals who meet the definition of neither LTNP nor P.

Individual participants with earlier designations in the literature are as follows: LTNP 1 (patient 1 [15, 38]); LTNP 2 (LTNP AD [22]); LTNP 3 (LTNP DJ [22]); LTNP 4 (LTNP HP [22, 39]); LTNP 6 (LTNP PC [22]); and SP 6 (SP EP [22]).

Peripheral blood mononuclear cell (PBMC) separations. Anticoagulated blood (heparin or acid citrate dextran) was processed for PBMC within 6 h of venipuncture by using Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) density centrifugation.

HIV-1 cultures. Routine virus cultures combined ≥ 5 × 10⁶ infected PBMC with an equal number of phytohemagglutinin (PHA)-activated PBMC from a seronegative donor, and viral replication was monitored with an ELISA assay for p24 antigen. CD8 T cell depletions of PBMC were performed in selected cases by using immunomagnetic bead separation as per manufacturer’s protocols (Dynabeads; Dynal, Great Neck, NY). Numerous variations that were intended to increase the sensitivity of virus recovery have been applied to 1 LTNP in particular (LTNP 4), from whom no virus has yet been isolated. These techniques have included that described by Finzi et al. [40], which employs CD3/CD28 costimulation of CD4 T cells ex vivo.

Virus phenotype. Routine infection of the MT-2 cell line was performed as described in the 1997 AIDS Clinical Trials Group (ACTG) Virology Manual [41], and cultures were monitored for viral replication by syncytium formation and p24 production. The engineered human osteosarcoma cell lines expressing CD4 and 1 of the chemokine receptors, CCR1–5, or GHOST cell lines (CXC4R4) obtained through the NIH AIDS Research and Reference Reagent Program, contributed by V. Kewal Ramani and D. Littman [42], were used to define chemokine receptor usage. These cells were infected with primary virus isolates as per the protocol of Cecilia et al. [43]. Primary isolate stocks of known titer were used to infect 1 × 10⁶ PHA-activated PBMC of a single uninfected donor in 24-well microtiter plates. Inocula were equalized by TCID₅₀ or by p24 content, as indicated in the figures. Replication kinetics were measured by p24 antigen accumulation in culture supernatant. Cultures were fed twice weekly with half volume medium exchanges (RPMI-10% with fetal calf serum–10 U/mL interleukin-2).
Quantitative polymerase chain reaction (PCR) studies. Proviral DNA copy frequency was estimated by using a modification of the Amplicor HIV-1 test system (Roche Diagnostic Systems, Branchburg, NJ). This assay uses primers to detect HIV-1 gag sequences in DNA prepared from lysates of whole blood. Prior to amplification, this DNA extract was serially diluted 10-fold. The cellular equivalents of DNA within each of these dilutions were calculated from the complete blood cell count and differential. The HIV-1 copy frequency in the population of lymphocytes was then determined from the end point of detection. Controls were included in each assay, which ensured a sensitivity of detection of 5 copies of proviral DNA. Similar results were obtained from lysates of PBMC and whole blood when they were tested in parallel by using samples from single blood collections (data not shown).

The Amplicor detection system was further modified to a quantitative format in which 3–4 replicate PCRs were run at each dilution (2- to 4-fold series) of a limiting dilution assay (LDA) series. The DNA copy frequency was calculated by using the QUALITY computer program available at website http://ubik.microbiol.washington.edu/cbu/quality/quality.html [44]. The CBC and differential were used along with lymphocyte surface marker data to calculate cell numbers for each reaction in the dilution series. We included the external standards from the Virology Quality Assurance Laboratory of the ACTG in each assay and required that a sensitivity of 5 copies per reaction be shown for an assay to be considered valid. The QUALITY program uses the minimum $\chi^2$ method for LDA end point calculations and further modifies the method to allow for user-specified probability estimates for false-negative and false-positive reactions.

Quantitative viral RNA PCR was performed by using the Amplicor HIV-1 Monitor assay (Roche Diagnostic Systems), with strict adherence to the manufacturer’s protocol. The serum or EDTA-anticoagulated plasma specimens used in these assays were processed within 6 h of phlebotomy and were stored at −80°C. The “ultrasensitive” modification of this technique was used for selected LTNP samples.

Lymphocyte marker studies. Whole blood immunophenotypic analyses were performed with fluorochrome-conjugated monoclonal antibodies (Becton-Dickinson, Mountain View, CA) and flow cytometry (FACScan, Becton Dickinson).

Cytotoxic T lymphocyte assays. Primary CTL effector (CTLe) frequencies were measured by using freshly isolated PBMC in an LDA format, as described elsewhere [23]. Target cells were autologous B-lymphoblastoid cell lines (BLCLs). Recombinant vaccinia viruses were provided by Dennis Panicali and Gail Mazzara (Therion Biologics, Cambridge, MA). These included vAbT 141 (expresses HIV-1inm gag p55) and vAbT 299 (expresses HIV-1inm gp160 with no signal sequence).

Secondary CTL precursor (CTLP) frequencies were determined as described elsewhere [45]. LDAs were performed with nonspecific stimulation of fresh PBMC or PBMC cryopreserved with OKT3. Irradiated autologous, PHA-stimulated PBMC were used as feeder cells. Chromium release assays were performed 7–14 days after stimulation.

The maximum likelihood method was used for CTL frequency calculations by using a cutoff 3 SD above the mean for spontaneous $^{51}$Cr release (statistical program kindly provided by S. Kalams, Massachusetts General Hospital, Boston). CTL frequency was taken to be the difference between frequencies calculated for activity against BLCL targets infected with recombinant vaccinia virus expressing HIV-1 antigen and activity against BLCL targets infected with control vaccinia. HIV-1–specific CTL frequency was expressed in terms of CTL number per 10$^6$ CD8-positive T lymphocytes.

CCR5 genetic analysis. The procedure of Huang et al. [46] was used to detect the 32-bp deletion polymorphism of the CCR5 alleles. Genomic DNA was extracted from 2 × 10$^7$ BLCL by using a DNA/RNA isolation kit (Amersham Life Science, Piscataway, NJ). The resultant pellet was resuspended in 30 mL of RNase-free water and 2 mL used for each amplification. The primers CCR5c (5'-CAAAAAAGAGGTCTTCATACC-3') and CCR5d (5'-CCTGTGCCTCTTCTCTCATTTG-3') were used at 20 pmol per reaction. Thermocycler settings consisted of 40 cycles: 5 cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C and 35 cycles of 30 s at 94°C, 30 s at 60°C, and 45 s at 72°C. This produces wild-type amplicons of 189 bp and deleted amplicons of 157 bp. These were analyzed on a 4% Metaphor agarose gel (FMC Bioproducts, Rockland, ME). Genetic analyses of the CCR2 and SDF-1 alleles of the LTNPs were performed as described elsewhere [6,7].

Lymphoproliferative assays. Freshly isolated PBMC of the LTNPs were tested for proliferative responses to HIV-1 p24 and gp160 by using an assay adapted from Rosenberg et al. [23]. Triplicate aliquots of 1 × 10$^5$ freshly isolated PBMC were incubated with HIV-1 recombinant antigens (p24 or gp160; Protein Sciences, Meriden, CT) at 5 mg/mL, baculovirus control protein, tetanus toxoid, or PHA in RPMI medium containing 10% human AB serum. Proliferation was measured by means of [3H] thymidine incorporation 6 days after stimulation. The stimulation index (SI) for HIV-1 antigens was calculated as the ratio of the mean counts per minute (cpm) of cells stimulated with viral antigen over the cpm of cells stimulated with the control protein.

Statistical methods. Kaplan-Meier survival analyses were performed with data censored as of December 1997. The relationships between CTL frequencies, virus titers, and absolute CD4 T cell counts were examined as described elsewhere [23] by using Pearson’s correlation coefficient (r) [47]. Significance of the correlation coefficient was assessed with Fisher’s transformation. Multiple regression analysis was applied to determine the relative strengths of relationships between continuous variables. The mean CTL frequencies and levels of virus measured by PCR for subgroups defined by CD4 T cell loss were compared by using Student’s t test. Statistical approaches for quantitative HIV-1 DNA PCR and CTL frequencies are described above. The software package StatView version 4.0 (Abacus Concepts, Berkeley, CA) was used for all calculations.

Results

General description of the cohort and its subgroups. This hemophilia cohort overall has shown a pattern of disease progression similar to that of other cohorts [48, 49]. In a Kaplan-Meier survival analysis, the median time to AIDS (1993 definition) was 9.1 years, assuming the year of infection to be 1982 for all individuals who were seropositive on study entry. This assumption is based on the observation that 76% of cohort
members studied by 1983 were seropositive and the remainder of infected individuals seroconverted by 1986.

The rates of infection with hepatitis B and C viruses (HBV and HCV) in the HIV-1–infected members of the cohort were 98.5% and 97.0%, respectively. Each of the cohort subgroups had a similar seroprevalence of HBV and HCV, and all LTNPs were infected with both HBV and HCV.

The CD4 T cell profiles of LTNPs (n = 7) have remained similar to those of age-matched seronegative (S-N; n = 37) members of the cohort (figure 1). The downward slope of the profiles of these 2 subgroups is primarily a manifestation of the youngest members of each group. Young children have higher absolute lymphocyte concentrations than those of adults, a difference that persists at significant levels for the first few years of life [50]. The youngest LTNP was born in 1979 and had a baseline CD4 T cell count ≥2500/μL when first measured in 1984. In 1997, at the age of 18 years, his average CD4 T cell count was 810/μL, with a CD4:CD8 ratio of 1.0. The early and gradual reductions in CD4 T cells observed in the LTNPs and seronegative members of the cohort were distinct from the declines in CD4 T cells observed in the other subgroups (figure 1).

Consistent with previous observations that age at infection is correlated with rate of disease progression [51], we found that rapid disease progression was associated with older age near the time of seroconversion. At the beginning of the study, in 1983, the ages of cohort members ranged from 0.6 to 70.9 years. There was no significant difference in the mean ages of seropositives and seronegatives (ages in 1983 were 22.4 and 23.4 years, respectively). Infected individuals with nonprogressive infection or slow disease progression (subgroups LTNP and SP) were significantly younger compared with those with rapid disease progression (mean ages in 1983 were 17.8 vs. 26.4 years, respectively; P < .001, Student’s t test).

Clinical events. The LTNPs in this cohort were identified in 1992, ~10 years after infection. Despite never having received antiretroviral treatment, they had maintained normal levels of CD4 T cells over this time period. During the subsequent 5 years of follow-up, 1 LTNP (LTNP 5) died of complications related to hepatic failure (in June of 1997) attributed to chronic

![Figure 1](https://i.imgur.com/3Q5Q5Q.png)

**Figure 1.** CD4 T cell profiles of cohort subgroups showing longitudinal measurement of CD4 T cell concentrations. Mean and standard errors are shown for each year. Figure legend indicates number in each subgroup contributing data for each year.
hepatitis C infection. LTNP 5 had normal CD4 T cell counts in the months prior to his death (range during 1996–1997, 510–680/μL; CD4 percentage, 33%). The last CD4 T cell concentration in 1997 of LTNP 1 fell below the levels defining this group (absolute CD4 count of 410/μL; CD4 percentage, 20%). It is noteworthy that at the time of this measurement, LTNP 1 remained asymptomatic and had a plasma virus RNA concentration <50 copies/mL. This individual has been discussed in greater detail in another report with more recent data included [38]. Through 1997, all other LTNPVs maintained values that met the criteria for nonprogression set in 1992.

LTNPs are defined in part by a lack of signs or symptoms attributable to HIV-1 infection. Individuals with hemophilia often have chronic conditions that may result in nonspecific signs or symptoms (e.g., chronic hepatitis C virus infection). In some cases these findings may be difficult to distinguish from disease owing to HIV-1. Nonetheless, the LTNPs identified in this cohort have never shown the relatively early features of HIV-1 disease such as generalized lymphadenopathy, oral thrush, or dermatomal zoster.

Chemokine and chemokine receptor polymorphism. Polymorphisms in the alleles of CCR5, CCR2, and SDF-1 have been associated with delayed disease progression [6, 7, 52]. To assess the role of these host factors in the LTNPs of this cohort, we first examined the CCR5 genotypes of members of the different subgroups. Host characteristics that are correlated with delayed disease progression include heterozygosity for a CCR5 allele with a 32-bp deletion (CCRSD32) [6]. Overall, the CCR5D32 allele frequency in this cohort was found to be similar to that of populations of Western European descent (11.8%) [53]. In this cohort, only 1 seronegative individual who was homozygous for the deletion variant was identified. Only 1 of the 7 LTNPs (LTNP 5) was heterozygous for this allele. Thus, the CCR5D32 allele frequency in these LTNPs was 7.1%. SPs exhibited a trend toward a higher allele frequency than Ps (13.4% vs. 7.1%, respectively; P > .05).

Other polymorphisms that have been associated with delayed disease progression include CCR2-64l and SDF-1-3A [6, 7]. Two LTNPs (1 and 4) were heterozygous for SDF-1-3A, and 1 LTNP (7) was heterozygous for both SDF-1-3A and CCR2-64l.

Virus load. Several different measurements were used to assess virus load. Stored serum or plasma samples were used to test viral RNA concentrations over the course of infection for LTNPs and similar numbers of individuals from the SP and P subgroups. The individuals in the comparison groups were selected randomly. Levels of circulating free virus in plasma of LTNPs were significantly lower than those in plasma of SPs and Ps at all time periods between 1984 and 1997 (figure 2). In fact, the majority (30/41 assays) of viral RNA levels in LTNPs were below the quantitation limit of this assay. Mean plasma viral RNA levels for the selected individuals from each subgroup diverged early during infection (before treatment be-

![Figure 2. Plasma viral RNA profiles of cohort subgroups showing longitudinal measurement of plasma viral RNA concentrations of selected members of each subgroup. Mean and standard errors are shown for 2-year intervals. Mean plasma viral RNA calculations used the quantitation limit of the assay (400 copies/mL) for samples in which RNA levels were not detectable.](https://academic.oup.com/jid/article-abstract/180/6/1790/867050)
by using standard culture techniques between 1987 and 1997 were 28% for the LTNP, 81% for the SPs, and 88% for the Ps. Rates of recovery from some of the progressing members of this cohort have been lower in recent years with the advent of potent antiretroviral therapies (data not shown). For selected LTNP with repeatedly negative virus cultures, CD8 depletions of PBMC were performed prior to culture to remove the suppressive effects of these cells on virus isolation. The only isolate from LTNP 1 was recovered by this technique. For LTNP 4, several modifications of cultures were attempted on each visit. These included CD8 T cell depletions, CD3/CD28 stimulations, SCID mouse engraftments with CD4 T cells, coculture with T cell lines expressing HIV-1 Tat, and coculture with autologous HTLV-1–transformed CD4 T cell lines, among other variations. For this individual there has been a total of >70 culture attempts on 21 study visits and not a single successful HIV-1 recovery. LTNP 4 has remained strongly positive for HIV-1 antibodies by Western blot despite these low virus loads [39].

Replication of viral isolates from LTNP. As viral stocks were being prepared for phenotypic characterization, it was noted that the primary isolates of the LTNP in this cohort seemed to replicate more slowly and to lower levels than the isolates of Ps (figure 3A). Replication kinetics of titered primary isolates were measured in “batch” assays in which PBMC from a single donor were infected with equal amounts of p24 antigen (5 ng; figure 3B) or equivalent infectious units (15 TCID_{50}; figure 3C). The isolates chosen for comparison of replication kinetics of LTNP isolates were selected randomly. They include 2 NSI isolates (from SP 1 and SP 4) and 2 SI isolates (from SP 6 and P 6). At the time of the experiment shown, titered stocks of LTNP 3 and LTNP 7 were not available. Repeated experiments have shown that the isolate from LTNP 7 replicates with kinetics similar to those of the LTNP 1 isolate, and the isolate from LTNP 3 replicated with kinetics similar to the LTNP 2 and 6 isolates (data not shown).

Virus isolated at two time points from LTNP 5 showed unusually slow replication kinetics even for LTNP viruses (figure 3A). Isolates from both time points failed to achieve concentrations in culture supernatants that would allow controlled comparisons with other virus strains. An attempt was made to expand this isolate into a high titer stock. Cultures were initiated for the LTNP 5 virus and 2 others from SPs concurrently, by using the same PHA-stimulated donor PBMC. Although the SP viruses accumulated p24 antigen in culture supernatant to levels well >100 ng/mL within 10 days, the virus from LTNP 5 never surpassed a level of 5 ng/mL, despite maintenance in culture for 35 days with weekly addition of 5 million PHA-stimulated donor PBMC from various donors. Independent subcultures of this virus have shown similar results. Further analysis of this and other LTNP viruses will be the subject of another report (present authors, unpublished data).

It was noted that infectious titers of LTNP virus stocks for a given concentration of p24 antigen tended to be lower than similarly derived stock cultures of individuals with progressive disease (figure 3C). When inocula were standardized by p24 content, large differences in levels of replication were observed between viruses of LTNP and Ps (figure 3B). When inocula were standardized by infectious dose, these differences were less pronounced. However, 2 of 3 isolates from LTNP grew more slowly and to lower levels than did viruses of individuals with progressive disease, despite inoculation with >10 times the p24 content (figure 3C).

Further phenotypic characterization of these virus isolates has shown all to be NSI in the MT-2 assay with no detectable replication in this T cell line. All failed to grow in various T cell lines, including CEMx174, C8166, Jurkat, HUT78, and A301 T cell lines. Characterization of coreceptor use by LTNP virus isolates by using the GHOST cell lines expressing CD4 and various chemokine receptors has shown all 6 isolates to use CCR5 exclusively (table 1).

Lymphocyte surface markers. Although CD4 T cell counts remained in the normal range (figure 1), 3 of 7 LTNP had an

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<th>Table 1. Summary of long-term nonprogressor (LTNP) virology.</th>
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<td><strong>Plasma viral RNA, copies/mL</strong></td>
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* Routine assay.  
+ Ultrasensitive assay. ND, not done.  
- Semiquantitative and quantitative.  
> Quantitative.  
\* No. of study visits with positive cultures over the total number of study visits when virus isolation was attempted.  
† The only positive culture was after CD8 T cell depletion.
Figure 3. Comparison of replication kinetics of virus isolates from long-term nonprogressors (LTNPs) and cohort members with disease progression. A, p24 antigen accumulation in culture supernatant of viral stock preparations. Ten cultures (viruses from 4 LTNPs [2, 5, 6, 7], 4 slow progressors [SPs; 1, 4, 6, 7], and 2 progressors [Ps; 5, 6]) were initiated in a batch on the same donor cells. Input inocula were not standardized. B, p24 antigen accumulation in supernatant of cultures inoculated with 5 ng of p24 antigen of each virus isolate. Seven cultures (viruses from 3 LTNPs [1, 2, 6], 3 SPs [1, 4, 6], and 1 P [6]) were initiated concurrently on the same donor cells. The measured p24 content of each inoculum (picograms added) is indicated by additional points on assay day 0. C, p24 antigen accumulation in supernatant of culture inoculated with 15 TCID50 of each virus isolate. Seven cultures (viruses from 3 LTNPs [1, 2, 6], 3 SPs [1, 4, 6], and 1 P [6]) are presented as in panel B. The measured p24 content of each inoculum (pg added) is indicated by additional points on assay day 0. Symbols are as in panel B. Virus stocks differed with respect to the no. of infectious units (TCID50) per nanogram of p24 antigen. For the titrated isolates shown, the measured TCID50 values/ng p24 were as follows: LTNP 1, 1; LTNP 2, 3; LTNP 6, 3; SP 1, 154; SP 4, 48; SP 6, 28; P 6, 80.

inversion in the CD4/CD8 ratio (table 2). Compared with seronegative members of the cohort, LTNPs maintained greater numbers of circulating CD8 T cells over the years of observation. LTNPs appeared also to have higher absolute CD8 T cell counts than members of this cohort with progressive disease, although this difference is less pronounced (figure 4A).

LTNPs maintained increased numbers of activated (HLA-DR–expressing) CD8 T cells over the past 8 years of the study (figure 4B). Although HIV-1–seronegative cohort members show some expansion of activated CD8 T cells (thought to be the result of chronic exposure to hepatitis viruses and exposure to alloantigen in factor concentrates), the LTNPs have usually shown values similar to those observed in Ps. The levels of other subpopulations of activated CD8 T cells (specifically CD8+CD28–DR+ and CD8+CD57+ T cells) showed trends similar to that of CD8+DR+ T cells, but without clear separation between the subgroups in this cohort (data not shown).

HIV-1–specific CTLe activity in freshly isolated cells. Freshly isolated effector cells that kill target cells presenting HIV-specific epitopes (especially epitopes of HIV-1 Gag antigen) have been detectable, often at high levels, in most members of this hemophilia cohort with progressive HIV-1 disease [45, 54]. We have shown previously that significant inverse relationships exist between Gag-specific CTLe frequencies and subsequent CD4 T cell loss, as well as between Gag-specific CTLe activity and cell-associated virus load [23].

High levels of CTLe activity were persistent in only 1 of 6 LTNPs (1) when repeat studies at multiple time points were performed. In all others, CTLe activity has been positive only intermittently, and generally at low levels. Three individuals (LTNPs 1, 2, and 4) have had both Env- and Gag-specific CTLe activity on multiple time points. The other 4 LTNPs had intermittent Gag-specific CTLe activity. A summary of these immunological characteristics of the LTNPs is presented in table 2.

With an expansion of the database to include more measurements of CTLe in LTNPs, we repeated the analyses presented by Greenough et al. [23]. In that report we observed a significant inverse correlation between Gag-specific CTLe frequencies and cell-associated virus load as measured by DNA PCR. Given this result, we had expected that LTNPs would have high levels of CTLe activity in association with low levels of cell-associated viral DNA. Additional measurements of Gag-specific CTLe frequencies in LTNPs, however, seemed to weaken the association somewhat (complete database: correlation coefficient, −0.298; P = .066; LTNPs excluded: correlation coefficient, −0.367; P = .038; table 3). Possible reasons for this are discussed below.
HIV-1–specific CTLp frequencies in cohort subgroups. We observed a significant correlation between CTLp frequencies and CD4 T cell counts (table 3). This observation contrasts the previously reported finding that CTLe frequencies are not correlated with concurrently measured CD4 T cell concentrations [23]. The mean Gag-specific CTLp frequency observed in LTNPs was significantly higher than that of cohort members with progressive disease (772 vs. 120 CTLp/10^6 CD8 T cells, respectively; Student’s t test, P = .010; figure 5). Because other studies have similarly shown a loss of detectable HIV-specific CTLp in advanced disease [26, 55], we reexamined these groups, excluding all with low CD4 T cell counts, and found that the LTNPs still had significantly higher Gag-specific CTLp frequencies than progressing members of the cohort. This difference was apparent when LTNPs were compared with progressing cohort members who had either >300 or >400 CD4 T cells/μL at the time of CTLp measurement (figure 5). Env-specific CTLp frequencies showed a similar trend but did not reach statistical significance (data not shown).

There was a significant correlation between CTLp frequencies and cell-associated virus loads (table 3). Env-specific CTLp, but not Gag-specific CTLp frequencies were independently associated with viral DNA concentrations in a multiple regression analysis with CD4 T cell counts as a second independent variable. Finally, there was no relationship between primary CTLe and secondary CTLp frequencies, as is evident from their relationships with CD4 T cell concentrations.

Proliferative responses to HIV-1 antigens. Recently developed assays testing CD4-dependent proliferative responses to HIV-1 antigens have shown that individuals in whom viral replication is effectively suppressed (with highly active antiretroviral therapy during acute infection or in long-term nonprogressive infection) tend to have large SIs [25]. We have measured proliferative responses to p24 and gp160 in fresh PBMC of LTNPs 1–4, 6, and 7. Three of the 6 have had SIs >20 in response to p24 antigen. Responses to gp160 were minimal (SIs of 1.1–4.3; table 2).

Discussion

Long-term nonprogressive HIV-1 infection may result from a number of different possible circumstances. LTNPs may be infected by HIV-1 containing a nonrevertible gene defect or HIV-1 with all genes intact. An effective immune response may be observed after infection by a strain of virus that is growth impaired in vivo by virtue of a nonrevertible gene defect. Examples of this are found in reports of individuals infected with nef-defective forms of HIV-1 [15, 56]. An unusually effective immune response may be seen after infection by a virus without gene defects for various reasons: (1) the viral antigens may have epitopes that bind avidly to the host major histocompatibility complex (MHC) molecules and induce potent virus-specific T cell responses; (2) the host cells may have an inherent resistance to infection by the infecting strain of HIV-1; (3) an intact thymus, or other factors that raise the regenerative capacity of the host, may favor the development of a potent cellular immune response; or (4) the infecting virus strain, despite the presence of intact genes, may have revertible mutations that result in an unusually slow growth phenotype in vivo, allowing sufficient time after acute infection for the development a response capable of controlling replication and minimizing viral evolution. The persistence of strains of virus that are growth impaired may result from a nonrevertible gene defect in the infecting strain or from ongoing immune pressure that selects for viruses with altered gene sequences.

Our studies of LTNPs in this cohort of individuals with he-

### Table 2. Summary of long-term nonprogressor (LTNP) cellular immunology.

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<th>LTNP</th>
<th>MHC class I haplotype</th>
<th>CD4 cells/mL</th>
<th>CD4 : CD8 ratio</th>
<th>HLA DR, % CD8 T cells</th>
<th>CTLLea</th>
<th>CTLPb</th>
<th>LPAc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A2; B13,62; C3,6</td>
<td>680</td>
<td>0.53</td>
<td>53</td>
<td>Gag 137, Env 165</td>
<td>Gag 433, Env 522</td>
<td>Gag 36</td>
</tr>
<tr>
<td>2</td>
<td>A29,68; B44,49; C2,7</td>
<td>1213</td>
<td>1.18</td>
<td>37</td>
<td>Gag 10, Env 7</td>
<td>Gag 149, Env 10</td>
<td>Gag 33</td>
</tr>
<tr>
<td>3</td>
<td>A2; B44; C2,5</td>
<td>624</td>
<td>0.60</td>
<td>38</td>
<td>Gag 89, Env 7</td>
<td>Gag 2481, Env 1285</td>
<td>Gag 62</td>
</tr>
<tr>
<td>4</td>
<td>A2,3; B58,60; C3,7</td>
<td>1147</td>
<td>1.50</td>
<td>49</td>
<td>Gag 22, Env 7</td>
<td>Gag 1427, Env 26</td>
<td>Gag 32</td>
</tr>
<tr>
<td>5</td>
<td>A2,32; B18,65; C5</td>
<td>782</td>
<td>0.67</td>
<td>72</td>
<td>Gag 15, Env 16</td>
<td>Gag 186, Env 879</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>A1,2; B44,57; C5,6</td>
<td>1196</td>
<td>1.58</td>
<td>40</td>
<td>Gag 14, Env 4</td>
<td>Gag 138, Env 3</td>
<td>Gag 1,7</td>
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<tr>
<td>7</td>
<td>A3,31; B38,64; C5,12</td>
<td>721</td>
<td>1.11</td>
<td>29</td>
<td>Gag 105, Env 3</td>
<td>Gag 587, Env 22</td>
<td>Gag 1,3</td>
</tr>
</tbody>
</table>

NOTE. All values are averages from 1990–1997. CTLe, cytotoxic T lymphocyte effector; CTLp, cytotoxic T lymphocyte precursor; MHC, major histocompatibility complex.

a Gag/Env-specific. CTLe and CTLp data units are nos./10^6 CD8 T cells. CTLe frequencies <15 and CTLp frequencies <50 are considered negative.

b Stimulation index, Gag/Env-specific. ND, not done.
Figure 4. CD8 T cell profiles of cohort subgroups. A, longitudinal measurement of CD8 T cell concentrations of cohort subgroups shown with slow progressors and progressors combined. Means and standard errors are shown for each year. Figure legend indicates the number in each subgroup contributing data for each year.

B, longitudinal measurement of percentage of CD8 T cells expressing HLA-DR for cohort subgroups, as shown in panel A. Mean and standard errors are shown for each year. Figure legend indicates the number in each subgroup contributing data for each year.
Table 3. Virologic and immunologic correlates with cytotoxic T lymphocyte (CTL) activity.

<table>
<thead>
<tr>
<th>Variable 1a</th>
<th>Variable 2a</th>
<th>Correlation coefficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gag-specific CTL frequencyb</td>
<td>DNAc</td>
<td>-2.298</td>
<td>.066</td>
</tr>
<tr>
<td>Gag-specific CTL precursor frequencyd</td>
<td>CD4 T cell countd</td>
<td>+4.78</td>
<td>.013</td>
</tr>
<tr>
<td>Gag-specific CTL precursor frequencyd</td>
<td>Viral DNA</td>
<td>-4.32</td>
<td>.030</td>
</tr>
</tbody>
</table>

NOTE. CTLe, CTL effector; CTLp, CTL precursor.
a Calculations of Pearson correlation coefficient and Fisher’s transformation use log10 transformation of all variables except CD4 T cell count.
b Units for CTL frequencies: no./10^6 CD8 T cells.
c Units for viral DNA: copies/10^6 CD4 T cells.
d Values when long-term nonprogressors

e Units for CD4 T cells: no./μL.

mophilia have provided a comprehensive evaluation of viral and host characteristics that distinguish this group from individuals with progressive disease. We have found LTNPs to be a group of individuals with unusually low virus loads and highly responsive and effective immune mechanisms. It appears that LTNPs have managed to achieve control of the infecting strains of HIV-1 such that the regenerative capacities of the host are able to keep pace with CD4 T cell destruction for more than a decade.

Viruses that we have isolated from LTNPs tend to replicate slowly, but, as yet, only 1 individual in this cohort (LTNP 1) was found to have been infected with a virus containing a nonrevertible gene defect [15]. These findings are in accord with those of a number of other laboratories. In most detailed studies, it is unusual to find LTNPs with evidence of infection by a virus with nonrevertible gene defects [15, 17, 18, 21, 22, 23, 56]. This suggests that most LTNPs are infected with viruses that are capable of evolving into a more virulent phenotype. Certain host characteristics appear to control this process in the LTNPs.

One possible explanation for control of the disease process lies in the avidity of virus antigenic epitopes for the host MHC molecules and the responsiveness of T cell repertoire to these antigens. Potent HIV-1–specific cellular immune responses are demonstrable in the LTNPs of this cohort. We have found strong HIV-specific CD4 T lymphocyte proliferative responses in 3 of 6 LTNPs studied in this cohort. This is in accord with the strong CD4 T cell proliferative responses to viral antigens that have been observed in chronically infected individuals who control viral replication effectively in the absence of antiretroviral therapy [25]. The presence of high frequencies of CTLp directed against Gag and Env antigens in the LTNPs of this cohort is consistent with a highly responsive cellular immunity to HIV-1.

Previously, we have shown a significant inverse correlation between CTLe frequencies and both virus load and subsequent CD4 T cell loss over time in this hemophilia cohort [23]. In many other studies, high levels of virus-specific CTL activity have been associated with lower viral replication and/or delayed disease progression [23, 24, 26–29, 57–62]. Given these relationships, it would follow that most LTNPs in this cohort would have persistently expanded CTLe populations. However, in most LTNPs we have found that CTLe activity is observed in freshly isolated PBMC only intermittently and generally at low levels. We hypothesize that this occurs because LTNPs have very low levels of viral replication and thus insufficient antigenic stimulation to expand CTLe to levels that are detectable by chromium-release assays.

In other members of this cohort with progressive HIV-1 infection, higher levels of viral replication result in maximally expanded populations of HIV-1–specific CTLe, which show an inverse relationship with viral DNA load and CD4 T cell loss [23].

Despite the low levels of plasma viral RNA in LTNPs and the relatively low levels of virus-specific CTLe activity, it is noteworthy that all LTNPs have high levels of expression of the activation marker HLA-DR on CD8 T cells. This suggests that a level of chronic immune stimulation exists in these individuals, even when viral RNA assays fail to show detectable levels of antigen production.

Aside from HIV-1–specific immune responses, the relative Figure 5. Comparison of Gag-specific cytotoxic T lymphocyte precursor (CTLp) frequencies of long-term nonprogressors (LTNPs) and progressors. Mean Gag-specific CTLp frequencies for LTNPs and progressors are shown with standard error. The progressors were randomly selected members of the cohort for whom cryopreserved cells were available for comparison of CTLp with cytotoxic T lymphocyte effector (CTLe) on a single sampling date.
susceptibility of the host cells to infection may contribute to the rate of disease progression. The identification of chemokine receptors as coreceptors for HIV-1 entry into cells has defined certain host factors that may play a role [6, 7]. The frequency of the $\text{CCR5D32}$ mutant allele is disproportionately high in most studies of LTNPs [63, 64]. However, we found only 1 LTNP in this cohort to be heterozygous for this mutation. Studies of the $\text{CCR2}$ and $\text{SDF-1}$ alleles of these LTNPs similarly fail to reveal a common genotype associated with nonprogressive infection. Although not present in the majority of LTNPs, these genetic polymorphisms that influence levels of expression of chemokine receptors on host cells may still contribute to a delay in disease progression in a given individual as part of a multifactorial process. The presence of both $\text{SDF-1-3.4}$ and $\text{CCR2-64l}$ alleles in LTNP 7 raises the possibility that additive effects may help to explain low levels of viral replication in vivo in some individuals.

Certain MHC haplotypes have been associated with more rapid or delayed disease progression [65]. Class I alleles that have been associated with delayed progression to AIDS (HLA-B57, A32, and B18) are present in a number of LTNPs in this group, whereas only 1 individual has alleles associated with more rapid progression to AIDS (HLA-B49 and -A29, found in LTNP 2; table 2). Heterozygosity of class I alleles has also been associated with slower disease progression [66] but was not a uniform finding in this group of LTNPs (table 2). As with chemokine/chemokine receptor studies, it can be concluded that no particular MHC class I allele (or combination of alleles) is found in the majority of LTNPs, but the allele(s) may have a contributing role in individual examples of nonprogressive HIV-1 infection.

Finally, age at the time of infection was found to correlate with disease progression in this cohort study. This supports the possibility that thymic integrity may play a role in the phenomenon of long-term nonprogressive HIV-1 infection.

We have isolated slow-growing viruses from the LTNPs $>10$ years after they were infected. Persistence of the slow-growth phenotype may be caused by the maintenance of strong selective immune pressure against more rapidly growing strains. Our studies of LTNP 4 have led us to the hypothesis that immune activity directed toward Nef epitopes has resulted in an unusually high frequency of defective nef alleles over time. Intact nef alleles appear to have been present in this LTNP at the earliest time points studied [22]. It remains to be defined to what extent revertible defects and slow-growth phenotype in the infecting strains allowed unusually effective immune control to develop in the LTNPs of this cohort.

In conclusion, we have found clear evidence of potent HIV-1–specific immune responses in all LTNPs in association with viruses that tend to replicate slowly in vitro and in vivo. The extent to which altered gene sequences associated with slow growth evolved over time, caused by selective immune pressures, or existed in the infecting strain remains to be defined for most of these individuals. The uniform presence of a nonrevertible viral gene defect has been shown in only 1 LTNP in this cohort to date. Individual LTNPs have cellular markers that may play a role in limiting viral replication and disease progression, but no host genotype has yet been found to universally and specifically correlate with nonprogressive infection. With these ongoing studies of LTNPs, we endeavor to define the characteristics of virus and host that result in control of viral replication and pathogenesis.

Acknowledgments

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References


